Catecholamine-dependent -adrenergic signaling in a pluripotent stem cell model of takotsubo cardiomyopathy

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Abstract: BACKGROUND: Takotsubo syndrome (TTS) is characterized by an acute left ventricular dysfunction and is associated with life-threating complications in the acute phase. The underlying disease mechanism in TTS is still unknown. A genetic basis has been suggested to be involved in the pathogenesis. OBJECTIVES: The aims of the study were to establish an in vitro induced pluripotent stem cell (iPSC) model of TTS, to test the hypothesis of altered -adrenergic signaling in TTS iPSC-cardiomyocytes (CMs), and to explore whether genetic susceptibility underlies the pathophysiology of TTS. METHODS: Somatic cells of patients with TTS and control subjects were reprogrammed to iPSCs and differentiated into CMs. Three-month-old CMs were subjected to catecholamine stimulation to simulate neurohumoral overstimulation. We investigated -adrenergic signaling and TTS cardiomyocyte function. RESULTS. Enhanced -adrenergic signaling in TTS-iPSC-CMs under catecholamine-induced stress increased expression of the cardiac stress marker NR4A1; cyclic adenosine monophosphate levels; and cyclic adenosine monophosphate-dependent protein kinase A-mediated hyperphosphorylation of RYR2-S2808, PLN-S16, TNI-S23/24, and Cav1.2-S1928, and leads to a reduced calcium time to transient 50% decay. These cellular catecholamine-dependent responses were mainly mediated by -adrenoceptor signaling in TTS. Engineered heart muscles from TTS-iPSC-CMs showed an impaired force of contraction and a higher sensitivity to isoprenaline-stimulated inotropy compared with control subjects. In addition, altered electrical activity and increased lipid accumulation were detected in catecholamine-treated TTS-iPSC-CMs, and were confirmed by differentially expressed lipid transporters CD36 and CPT1C. Furthermore, we uncovered genetic variants in different key regulators of cardiac function. CONCLUSIONS. Enhanced -adrenergic signaling and higher sensitivity to catecholamine-induced toxicity were identified as mechanisms associated with the TTS phenotype. (International Takotsubo Registry [InterTAK Registry] [InterTAK]; NCT01947621).

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Catecholamine-Dependent β-Adrenergic Signaling in a Pluripotent Stem Cell Model of Takotsubo Cardiomyopathy

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ABSTRACT

BACKGROUND Takotsubo syndrome (TTS) is characterized by an acute left ventricular dysfunction and is associated with life-threating complications in the acute phase. The underlying disease mechanism in TTS is still unknown. A genetic basis has been suggested to be involved in the pathogenesis.

OBJECTIVES The aims of the study were to establish an in vitro induced pluripotent stem cell (iPSC) model of TTS, to test the hypothesis of altered β-adrenergic signaling in TTS iPSC-cardiomyocytes (CMs), and to explore whether genetic susceptibility underlies the pathophysiology of TTS.

METHODS Somatic cells of patients with TTS and control subjects were reprogrammed to iPSCs and differentiated into CMs. Three-month-old CMs were subjected to catecholamine stimulation to simulate neurohumoral overstimulation. We investigated β-adrenergic signaling and TTS cardiomyocyte function.

RESULTS Enhanced β-adrenergic signaling in TTS-iPSC-CMs under catecholamine-induced stress increased expression of the cardiac stress marker *NR4A1;* cyclic adenosine monophosphate levels; and cyclic adenosine monophosphate–dependent protein kinase A–mediated hyperphosphorylation of RYR2-S2808, PLN-S16, TNI-S23/24, and Cav1.2-S1928, and leads to a reduced calcium time to transient 50% decay. These cellular catecholamine-dependent responses were mainly mediated by β_1-adrenoceptor signaling in TTS. Engineered heart muscles from TTS-iPSC-CMs showed an impaired force of contraction and a higher sensitivity to isoprenaline-stimulated inotropy compared with control subjects. In addition, altered electrical activity and increased lipid accumulation were detected in catecholamine-treated TTS-iPSC-CMs, and were confirmed by differentially expressed lipid transporters *CD36* and *CPT1C*. Furthermore, we uncovered genetic variants in different key regulators of cardiac function.

CONCLUSIONS Enhanced β-adrenergic signaling and higher sensitivity to catecholamine-induced toxicity were identified as mechanisms associated with the TTS phenotype. (International Takotsubo Registry [InterTAK Registry] [InterTAK]; NCT01947621) (J Am Coll Cardiol 2017;70:975–91) © 2017 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

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ABBREVIATIONS AND ACRONYMS

β-AR = β-adrenergic receptor

cAMP = cyclic adenosine monophosphate

CD36 = fatty acid translocase membrane protein

cGMP = cyclic guanosine monophosphate

CGP = β_1-adrenergic receptor blocker CGP 20712A

CM = cardiomyocyte

Epi = epinephrine

FRET = Förster resonance energy transfer

ICI = β_2-adrenergic receptor blocker ICI 118551

iPSC = induced pluripotent stem cell

Iso = isoprenaline

MEA = multielectrode array

NR4A1 = nuclear receptor subfamily 4 group A member 1

PKA = protein kinase A

T50 = time to transient 50% decay (from maximum)

TTS = Takotsubo syndrome

WES = whole-exome sequencing

Takotsubo syndrome (TTS) is an increasingly diagnosed disease with symptoms similar to acute myocardial infarction, including clinical symptoms, electrocardiogram changes, and cardiac biomarkers, usually in the absence of obstructive coronary artery stenosis (1,2). The syndrome is often triggered by emotional or physical stressors (3,4). TTS is life-threatening, with 22% of patients experiencing serious in-hospital complications, such as ventricular tachycardia, cardiogenic shock, or death (2). Even long-term follow-up is not completely benign, as the rate of recurrence in TTS is 2%, and the major adverse cardiac and cerebrovascular event rate is 10% per patient-year (2).

Several hypotheses have been proposed to describe the pathomechanisms (5,6); however, the exact mechanism leading to TTS is still under debate. Detailed mechanistic studies are difficult to perform due to the lack of a reliable TTS model that translates to humans. Adrenergic overstimulation seems to play an important role in TTS, and it has been suggested that heart function can be damaged by excessive catecholamine release (7). This indicates that a normally protective mechanism might fail in these subjects. Several familial cases of TTS have been described in published reports (8), although it is unclear to date whether this disease has a genetic component. Small studies in individual patients have reported an association between TTS and several genes, including Bcl-2-associated athanogene 3 (*BAG3*) (9).

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A big hurdle in understanding the molecular and cellular physiology of TTS has been the lack of human cardiomyocyte (CM) culture models from patients with TTS. The availability of methods to generate induced pluripotent stem cells (iPSCs) and to direct their differentiation toward a CM phenotype overcomes this hurdle by enabling the creation of patient-specific TTS disease models. iPSC-CMs have been especially widely used to study hereditary cardiac conditions in vitro, including arrhythmic disorders or cardiomyopathies, with a robust correlation to predicted phenotypes (10). A genetic predisposition, although suggested (8), is less well established for TTS. We tested the hypothesis that TTS-iPSC-CMs phenocopy the disease in the dish, and thus allow for mechanistic studies to facilitate the development of novel therapeutic and protective strategies in patients with TTS.

METHODS

Details are available in the Online Appendix.

PATIENTS. Patients with TTS (not related to each other) presented here have been selected from the International Takotsubo Registry (NCT01947621) and fulfilled the Mayo Clinic Diagnostic Criteria for TTS (Online Table 1) (11). A total of 4 healthy donors were used as control subjects in this study (Online Table 1). One healthy control subject (control 1) is a 52-year-old postmenopausal woman. Two further control subjects are 25-year-old women (controls 2 and 3). One anonymous healthy woman control subject (FB2) was characterized and described in our previous study (12).

REPROGRAMMING OF SOMATIC CELLS INTO iPSCs. Fibroblasts (TTS patients 1, 2, 5 and 8; controls 2 and 3) or peripheral blood cells (control 1) were reprogrammed using nonintegrating systems, and high-quality iPSC clones were expanded. Cell lines were named 1-TTS-1, 1-TTS-2, 2-TTS-1, 5-TTS-1, 5-TTS-2, 8-TTS-1, and 8-TTS-2 from TTS patients 1, 2, 5, and 8; and 1-C-1, 1-C-2, 2-C-1, 2-C-2, 3-C-1, and 3-C-2 for cell lines from healthy controls 1, 2, and 3 (Online Table 2).

CHARACTERIZATION OF iPSC-CMs. iPSCs were differentiated into CMs using Wnt modulation (13) and subsequent metabolic selection (14). iPSC-CMs

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were cultured for 2 to 3 months and subjected to catecholamine stimulation with isoprenaline (Iso) or epinephrine (Epi), as indicated. CMs were then subjected to analyses of cytoplasmic cyclic adenosine monophosphate (cAMP) levels, protein kinase A (PKA)-specific phosphorylation of target proteins (ryanodine receptor type 2 [RYR2]-2808, phospholamban [PLN]-S16, troponin I [TNI]-S23/24, L-type calcium channel [Cav1.2]-S1928), calcium decay time (transient decay time to 50% [T50]), engineered heart muscle (EHM) for contractility measurements, expression of stress- and lipid transport-related genes, cardiac lipid accumulation, and beating frequency by multielectrode array (MEAs). Most of the experiments were performed under β-adrenergic receptor (β-AR)-subtype-specific conditions using inhibitors against β_1-ARs (100 nmol/l of CGP 20712A [CGP]) and/or β_2-AR (50 nmol/l of ICI 118551 [ICI]). Primers and antibodies used in this study are provided in Online Tables 3 and 4.

RESULTS

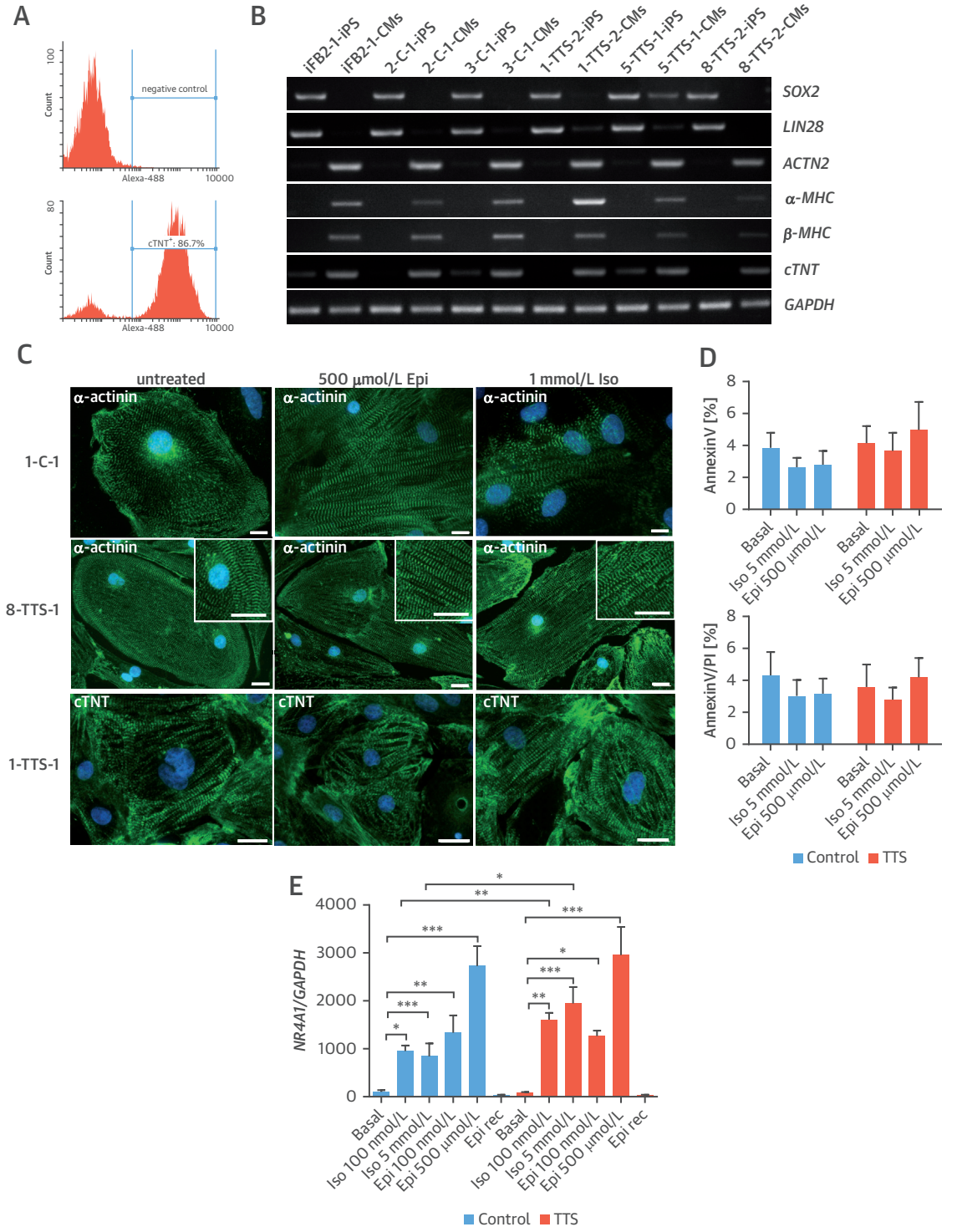
iPSCs GENERATED FROM PATIENTS WITH TTS SHOW PLURIPOTENT CHARACTERISTICS AND CAN BE DIFFERENTIATED INTO FUNCTIONAL CMs. The iPSC lines generated for this study showed typical human pluripotent stem cell characteristics (Online Figure 1). No differences in differentiation efficiency were observed between control and TTS-iPSCs. Using a directed differentiation protocol, we achieved a cardiomyocyte purity of >85% at culture day 60 (determined by flow cytometry analysis for cardiac troponin T [cTNT]) (**Figure 1A**). The CM cultures derived from both control- and TTS-iPSCs expressed high levels of cardiac-specific genes, such as α-actinin *(ACTN2),* α-myosin heavy chain *(α-MHC),* β-myosin heavy chain *(ß-MHC),* and *cTNT* at the mRNA level, and show α-actinin-, and cTNT-positive sarcomeric structural features (**Figures 1B and 1C**, Online Video 1).

BRIEF CATECHOLAMINE TREATMENT AS AN INDUCER OF STRESS. Exposure to high levels of catecholamines has been implicated in TTS (7). We therefore aimed to induce a TTS-related phenotype in iPSC-CMs after exposure to catecholamines (Iso [100 nmol/l to 5 mmol/l], and Epi [100 nmol/l to 500 μmol/l]), as described previously (6,7). These experimental conditions did not result in an irregular sarcomeric structure (**Figure 1C**) or apoptosis as analyzed by annexin V/propidium iodide (PI) flow cytometric assay (**Figure 1D**). We then analyzed the expression of cardiac stress-related genes in iPSC-CMs, including *NR4A1,* by quantitative PCR. *NR4A1* is strongly increased for all tested concentrations of Iso and Epi in both TTS-iPSC-CMs (16- to 19-fold for Iso; 13- to 30-fold for Epi) and control-iPSC-CMs (10- to 8-fold for Iso; 13- to 27-fold for Epi) (**Figure 1E**). After Iso stimulation, *NR4A1* expression was significantly greater in TTS-iPSC-CMs than in control-iPSC-CMs. These changes in *NR4A1* expression were reversed after a 3-week catecholamine washout period (**Figure 1E**, Epi rec).

EFFECTS OF CATECHOLAMINES ON β-ADRENERGIC SIGNALING. β-ARs activate the Gs/adenylate cyclase (ADCY) cascade, generating the second messenger cAMP. To analyze the activation of this pathway, we measured cAMP levels, PKA activity, and electrical activity in iPSC-CMs from TTS and control subjects. cAMP dynamics were measured by transduction of iPSC-CMs with an adenoviral construct expressing the Förster resonance energy transfer (FRET)-based cAMP sensor Epac1-camps under the control of the cytomegalovirus promoter (15). Control iPSC-CMs reacted to the β-AR agonist Iso (100 nmol/l), which demonstrated a clear increase in the cyan fluorescent protein (CFP)/yellow fluorescent protein (YFP) ratio and cytoplasmic cAMP levels (**Figures 2A and 2C**). There was no additional effect at greater Iso levels (1.18-fold increase at 1 mmol/l Iso) (**Figures 2A and 2C**). In contrast, TTS CMs reacted to higher Iso concentrations of 1 μmol/l (1.55-fold; $p = 0.0621$) and 1 mmol/l (1.98; $p = 0.0029$) with a further significant increase in the CFP/YFP ratio, and therefore higher cytoplasmic cAMP levels in TTS-CMs relative to control iPSC-CMs (**Figures 2B and 2C**). This suggests a higher catecholamine-dependent dynamic range in TTS-iPSC-CMs. As cAMP can be synthesized at low rates under basal conditions, we pretreated the iPSC-CMs with the unselective phosphodiesterase (PDE) inhibitor 3-isobutyl-1-methylxanthin (IBMX) in the absence of Iso. Under these experimental conditions, we detected no significant difference in FRET response in control and TTS-iPSC-CMs (Online Figures 2A to 2C). This suggests similar basal cytoplasmic cAMP levels in iPSC-CMs of patients with TTS compared to control subjects.

To further investigate β-adrenergic signaling in TTS-iPSC-CMs, we analyzed the activation of PKA by phosphorylation of its target sites RYR2-2808, PLN-S16, TNI-S23/24, and Cav1.2-S1928. Western blots of Iso- and Epi-treated iPSC-CMs showed significantly greater phosphorylation of PLN-S16, RYR2-S2808, and Cav1.2-S1928 in TTS-iPSC-CMs after catecholamine treatment (**Figures 2D to 2G**). TNI-S23/24 exhibited a trend toward enhanced phosphorylation

FIGURE 1 Brief Catecholamine Treatment as an Inducer for Stress in Generated iPSC-CMs



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in TTS compared with control iPSC-CMs (**Figure 2H**). Importantly, we found a significant 5.7-fold increase of PLN-S16 phosphorylation at a basal level without catecholamine treatment in TTS-iPSC-CMs (**Figure 2E,** Online Figures 2D and 2E). An additional Iso treatment resulted in a 13- (100 nmol/l) or 14-fold (5 mmol/l) greater absolute phosphorylation in TTS compared with control subjects (**Figures 2D and 2E**). After normalization, we found no significant differences in phosphorylation between TTS and control subjects (Online Figure 3C). PKA-specific RYR2-S2808 showed a 3.3- to 6.4-fold greater phosphorylation and Cav1.2-S1928 showed a 3.3- to 3.7-fold greater phosphorylation after Iso treatment (100 nmol/l, 5 mmol/l) compared with untreated cells from patients with TTS. In contrast, the phosphorylation of control-iPSC-CMs was only increased 1.2- to 3.0-fold for RYR2, and 1.5- to 1.6-fold for Cav1.2 under the same conditions (**Figures 2F and 2G**). The extracellular signal-regulated kinase (ERK), involved in cardiac structural remodeling and known to be activated by β-AR-dependent phosphorylation, was maximally phosphorylated in TTS after treatment with 100 nmol/l Iso or Epi. In contrast, control iPSC-CMs had reduced ERK phosphorylation compared with TTS iPSC-CMs (**Figure 2D**).

Similar results were obtained after Epi treatment of iPSC-CMs (Online Figures 3A, 3B, 3D, and 3E). We also quantified the basal protein expression of key downstream targets of β-AR signaling and calcium-handling proteins, and detected decreased expression of total PLN (Online Figure 2E). RYR2, TNI, phosphorylation of RYR2-S2808, and TNI-S23/24 (β-AR downstream partners) all showed no significant differences in protein expression, but exhibited a decreasing trend in unstressed TTS-iPSC-CMs toward levels detected in control-iPSC-CMs. The key calcium-handling proteins sarcoplasmic/endoplasmic reticulum calcium ATPase 2a (SERCA2a) and sodium-calcium exchanger 1 (NCX1) were similarly expressed under basal conditions in control- and TTS-iPSC-CMs (Online Figure 2E). Furthermore, expression of these proteins was not altered after catecholamine treatment in TTS- and control-iPSC-CMs (**Figure 2D**).

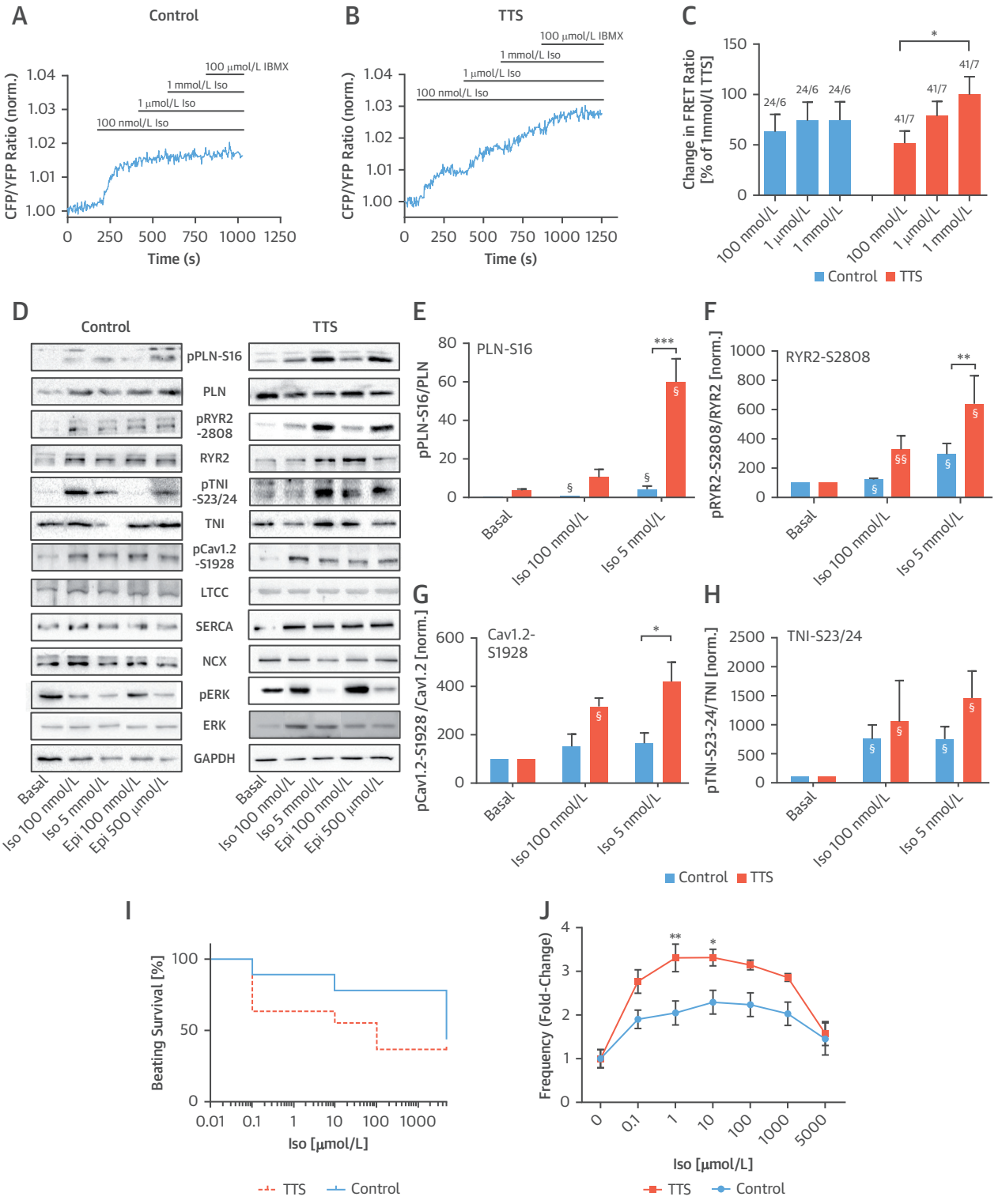
ALTERED ELECTRICAL ACTIVITY OF CATECHOLAMINE-TREATED iPSC-CMs FROM PATIENTS WITH TTS. As cAMP was shown to regulate chronotropic, inotropic, and lusitropic effects in the mammalian heart (16), we sought to analyze the effects of altered β-adrenergic signaling in the bioelectrical features of catecholamine-treated TTS-iPSC-CMs using the MEA system. In more than one-half of the analyzed TTS-iPSC-CMs, electrical activity was completely silenced at different Iso concentrations, which was observed in only a few control-iPSC-CMs under the same conditions (**Figure 2I,** Online Figure 4). However, unsilenced TTS-iPSC-CMs showed a significantly increased beating frequency in comparison with control-iPSC-CMs at 1 μmol/l Iso (3.3 ± 0.3 vs. 2 ± 0.3; $n = 7$ for TTS and 8 for control; $p = 0.002$) and 10 μmol/l Iso (3.3 ± 0.2 vs. 2.3 ± 0.3; $n = 7$ for TTS and 8 for control; $p = 0.04$) (**Figure 2J**), whereas there were no differences in basal beating frequencies between TTS and control subjects (Online Figure 4B). Of note, the beating frequency in control and TTS-CMs was suppressed when Iso concentrations exceeded 10 and 1 μmol/l, respectively (**Figure 2J**). These changes in electrical activity were completely reversible upon washout of Iso after 24 h (Online Figure 4).

ALTERED FUNCTIONALITY OF CALCIUM HOMEOSTASIS AND FORCE DEVELOPMENT IN PATIENTS WITH TTS. Due to Iso-dependent alterations in cAMP levels, PKA activity, and electrical activity in TTS, we sought to investigate calcium kinetics in iPSC-CMs. We

FIGURE 1 Continued

(A) Flow cytometry analysis of cTNT expression was performed at day 60 post-cardiac differentiation; 87% of $cTNT^+$ CMs were obtained after metabolic selection. **(B)** Six iPSC lines (representative of patients 1, 5, and 8, and control subjects 2, 3, and iFB2 [published previously]) express decreasing levels of the pluripotency markers *SOX2* and *LIN28* when differentiated towards CMs. Expression of the cardiac markers *ACTN2, α-MHC, β-MHC* and *cTNT* is strongly enhanced in differentiated iPSC-CMs (Online Video 1). **(C)** Three-month-old iPSC-CMs were stained with antibodies against the sarcomeric proteins α-actinin or cTNT with or without catecholamine treatment (2 h). **Scale bar:** 10 μm for α-actinin and 20 μm for cTNT. Representative images are shown from control and TTS cell lines. **(D)** Percentage of annexin V **(top)** or annexin V/propidium iodide–positive cells **(bottom)** with or without catecholamine treatment (2 h). Control: n = 4 differentiation experiments (1-control [1×], 2-control [1×], iFB2 [2×]); TTS: n = 3 differentiation experiments (1-TTS [1×], 5-TTS [1×], 8-TTS [1×]). **(E)** Expression of *NR4A1* mRNA in iPSC-CMs from control and TTS patients. IPSC-CMs were treated with catecholamines for 2 h. Control: n = 6 differentiation experiments (1-control [2×], 2-control [2×], iFB2 [2×]); TTS: n = 6 differentiation experiments (1-TTS [2×], 8-TTS [4×]). Mean ± SEM, *p < 0.05; **p < 0.01; ***p < 0.001 significant differences by 1-way ANOVA for each group (control, TTS) combined with the Student *t* test. Cells were counterstained with 4′,6-diamidino-2-phenylindole for nuclear localization **(blue) (C)**. *GAPDH* was used as internal control **(B and E)**. ANOVA = analysis of variance; CM = cardiomyocyte; cTNT = cardiac troponin T; Epi = epinephrine; *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase; iPSC = induced pluripotent stem cell; Iso = isoprenaline; mRNA = messenger ribonucleic acid; *NR4A1* = nuclear receptor subfamily 4 group A member 1; SEM = standard error of the mean; TTS = Takotsubo syndrome.

FIGURE 2 Increased Catecholamine-Induced β-Adrenergic Signaling in iPSC-CMs



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detected a significantly lower T50 in TTS-iPSC-CMs compared with control subjects in the absence (T50: 883.6 ± 30.3 ms vs. 1,018.3 ± 35.6 ms; adjusted $p = 0.006$) and presence of Iso stimulation (at 100 nmol/l and 1 mmol/l Iso) (**Figure 3A**). Of note, in TTS, lower Iso concentrations (100 nmol/l) were sufficient to significantly reduce T50 compared with control subjects (1 μmol/l) (Online Figure 5B, **Figure 4D**, blue bars). Furthermore, we found an increase in the amplitude of calcium transients (Ca^{2+}_I) in the TTS-iPSC-CMs compared with control subjects in unstimulated cells (6.4 ± 0.3 Ca^{2+}_I vs. 5.1 ± 0.2 Ca^{2+}_I; adjusted $p = 0.0007$) and under Iso stimulation of 100 nmol/l (Online Figure 5D).

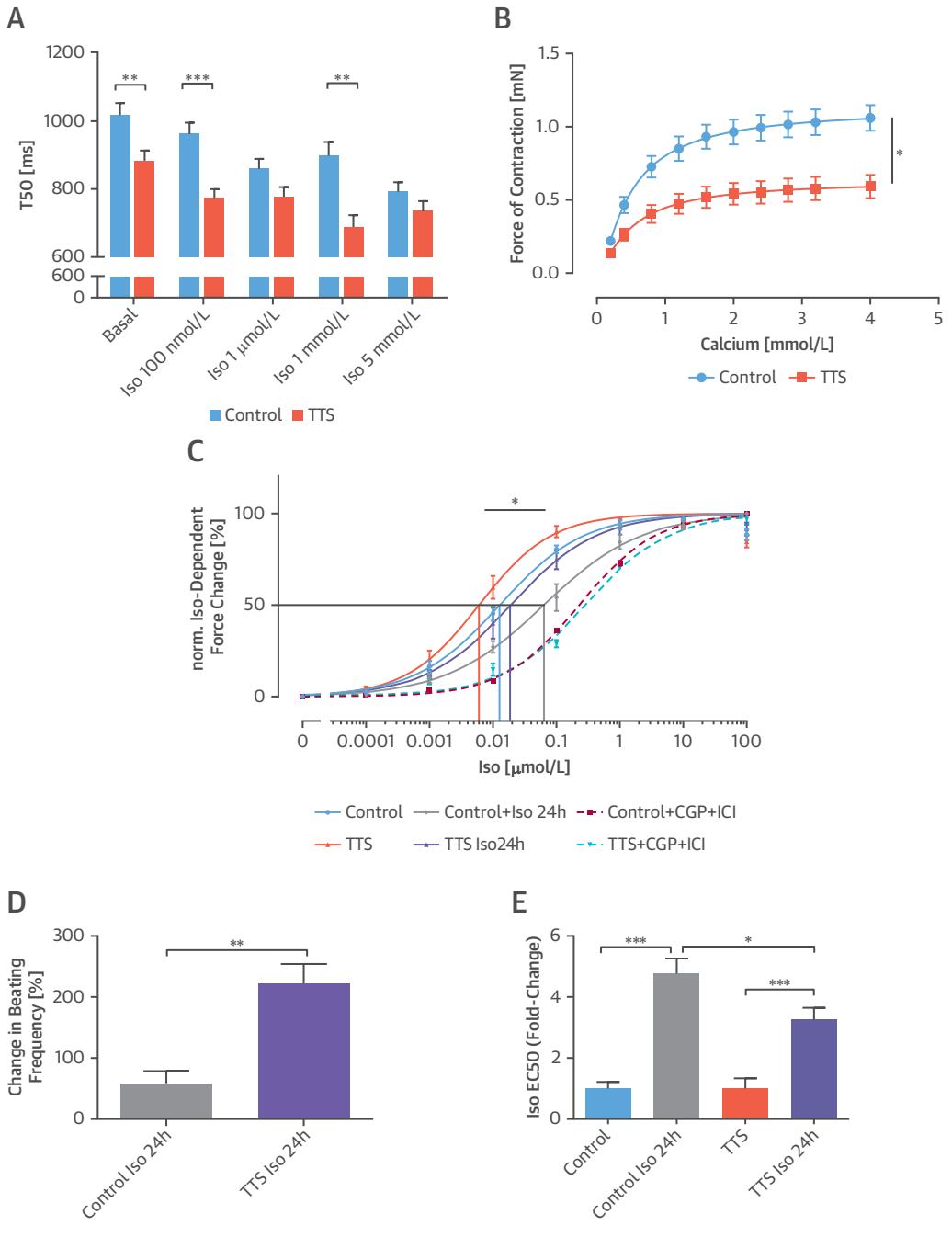
Engineered heart muscle (EHM) from TTS-iPSC-CMs (Online Video 2) showed an impaired absolute force of contraction in comparison with control EHMs (**Figure 3B**). In line with the calcium kinetics of single cardiomyocytes (**Figure 3A**), we observed faster relaxation times in TTS EHMs under baseline conditions and in the presence of 1 μmol/l Iso compared with control subjects (Online Figure 6A). Furthermore, we found higher sensitivity to adrenergic stimulation in TTS EHMs (Iso half-maximal effective concentration [EC_{50}]: 6 ± 1 nmol/l vs. 13 ± 2 nmol/l; $n = 10$ for TTS and 11 for control; $p < 0.05$), which was efficiently antagonized by the combination of the β-AR-inhibitors ICI (β_2-selective antagonist) and CGP (β_1-selective antagonist) (**Figure 3C**). Interestingly, not only was the sensitivity to acute Iso stimulation increased in TTS compared with control EHMs (**Figure 3C**), but chronic Iso stimulation for 24 h also resulted in significant differences between TTS and control EHMs. In line with the acute monolayer CM data (**Figure 2J**), we observed a greater change in beating frequency after 24 h Iso in TTS compared with control (222 ± 32% vs. 59 ± 19%; $n = 7$ for TTS and 5 for control; $p < 0.05$) (**Figure 3D,** Online Figure 6B). Furthermore, 24-h chronic stimulation resulted in a right shift of the acute Iso concentration curve, indicating rapid catecholamine desensitization in EHM. However, this shift was less pronounced in TTS EHMs in comparison with control EHMs (Iso EC_{50}: 19 ± 4 nmol/l vs. 64 ± 10 nmol/l; $n = 7$ for TTS and 5 for control; $p < 0.05$) (**Figures 3C and 3E**), indicating a lower degree of catecholamine desensitization in TTS-EHMs.

SELECTIVE β-ADRENERGIC SIGNALING IN TTS-iPSC-CMs. To further assess the contribution of specific β-AR subtypes in TTS-iPSC-CM signaling, cells were treated with ICI (β_2-antagonist), CGP (β_1-antagonist), or both. We examined cAMP generation; PKA activity by assessing the phosphorylation of RYR2, PLN, TNI, and Cav1.2; calcium fluctuation; and beating frequency by MEA measurements for antagonist-specific effects in TTS-iPSC-CMs. We found that β-AR activity was mediated by both β_1- and β_2-ARs in TTS-iPSC-CMs, as demonstrated by a significantly increased β_1-AR- and β_2-AR-mediated cAMP (ICI or CGP) at most Iso concentrations compared with control subjects (**Figures 4A and 4B,** Online Figures 7D to 7F).

FIGURE 2 Continued

(A and B) Representative cAMP-FRET traces from the Epac1-cAMP-FRET sensor in adenovirally transduced iPSC-CMs from patients with TTS and control subjects stimulated with increasing β-AR agonist concentrations (100 to 1 mmol/l Iso). The subsequent maximal FRET response was induced by the unselective phosphodiesterase (PDE) inhibitor 3-isobutyl-1-methylxanthine (IBMX) (100 μmol/l). **(C)** Quantification of the FRET experiments. β-AR stimulations led to stronger FRET responses in the cytosol in TTS-iPSC-CMs compared with control subjects at higher Iso concentrations of 1 mmol/l. The number of CMs and differentiation experiments are indicated above the **bars** (CMs/differentiation experiments). Control: $n = 24$ cells (2-control [$n = 2$], 3-control [$n = 7$], and iFB2 [$n = 15$]); TTS: $n = 41$ cells (1-TTS [$n = 15$], 5-TTS [$n = 16$], and 8-TTS [$n = 10$]). *$p < 0.05$ significant differences by 1-way ANOVA. **(D to H)** Activation of PKA shown by phosphorylation of its target sites RYR2-2808, PLN-S16, TNI-S23/24, Cav1.2-S1928. **(D)** Representative original Western blots of Iso- and Epi-treated (2 h) iPSC-CMs of control subjects (2-control) and patients with TTS (1-TTS). **(E)** Quantified absolute PLN-S16 phosphorylation is increased in TTS patients in comparison with control cells (control: $n = 3$ differentiation/Western experiments (2-control [$n = 2$], iFB2 [$n = 1$]); TTS: 4 differentiation/Western experiments (1-TTS [$n = 3$], 5-TTS [$n = 1$]) upon Iso. **(F to H)** Quantified and normalized RyR2-S2808-, Cav1.2-S1928-, and TNI-S23/24 phosphorylation is increased in patients with TTS in comparison with control cells ($n = 3$ to 7 differentiation experiments; for details see Online Table 5) upon Iso. Data are presented as mean ± SEM of the ratio of normalized phosphorylated protein over total protein. * or §$p < 0.05$; ** or §§$p < 0.01$; ***$p < 0.001$ significant differences by multiple Student *t* tests and the Sidak-Bonferroni method. §Significant differences of Iso-treated iPSC-CMs over basal conditions (analyzed by Student *t* test). **(I and J)** Electrical disturbances in iPSC-CMs of patients with TTS after catecholamine treatment. **(I)** Quantification of silenced electrical activity of iPSC-CMs of patients with TTS and control subjects presented as beating survival curve. TTS-CMs showed an earlier cessation of beating compared with control subjects. Control: $n = 9$ MEA experiments (1-control [$n = 6$], 2-control [$n = 1$], and iFB2 [$n = 2$]); TTS: $n = 11$ MEA experiments (1-TTS [$n = 8$] and 8-TTS [$n = 3$]). **(J)** Iso-dose-dependent frequency increase in iPSC-CMs at Iso concentrations up to 1 μmol/l (TTS) or 10 μmol/l (control). Higher Iso concentrations result in a decreased frequency. Control: $n = 8$ MEA experiments (1-control [$n = 6$], 2-control [$n = 1$], and iFB2 [$n = 1$]); TTS: $n = 7$ MEA experiments (1-TTS [$n = 6$] and 8-TTS [$n = 1$]). Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$; significant differences by 2-way ANOVA. β-AR = β-adrenergic receptor; cAMP = cyclic adenosine monophosphate; ERK = extracellular signal-regulated kinase; FRET = Förster resonance energy transfer; MEA = multielectrode array; NCX1 = sodium calcium exchanger 1; PKA = protein kinase A; SERCA2a = sarcoplasmic/endoplasmic reticulum calcium ATPase2a; other abbreviations as in **Figure 1**.

FIGURE 3 Iso-Dependent Calcium Homeostasis and Force Development in iPSC-CMs



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Additionally, increasing Iso-dependent FRET responses under ICI or CGP antagonism are observed in TTS (Online Figures 7D to 7F). Furthermore, PKA-promoted phosphorylation of RYR2, PLN, and Cav1.2 was mediated by both β_1-AR (ICI) and β_2-AR (CGP), or predominantly by β_1-ARs (Online Figure 8). Only PKA-dependent phosphorylation of TNI was shown to be mainly promoted by β_2-AR signaling in TTS (Online Figure 8). The contribution of both β_1- and β_2-ARs to the β-AR activity was confirmed by

MEA data showing Iso-dependent rising frequencies after ICI or CGP treatment in TTS, similar to cells without inhibitor (**Figure 4C**). In contrast, in control cells, the vast majority of Iso-dependent β-AR activity (cAMP generation, PKA-promoted phosphorylation of TNI and Cav1.2, and an Iso-dependent increase in beating rate) was mediated by β_2-ARs (CGP) (**Figures 4A to 4C,** Online Figures 7F and 8). Of note, the Iso-dependent reduction of calcium T50 is mainly mediated by β_1-ARs in TTS, whereas β_2-ARs are the main contributors in control iPSC-CMs (**Figure 4D**). Taken together, TTS-specific effects of antagonists were mainly mediated by β_1-ARs, but our findings indicate that β_2-ARs are involved as well.

Simultaneous inhibition of both β-AR subtypes (ICI + CGP) suppressed FRET changes, T50-changes (up to 1 μmol/l Iso in TTS), beating rate (up to 1 μmol/l Iso in TTS and control subjects), and PKA-specific phosphorylation, indicating that catecholamine-induced changes are mediated by β_1-AR, β_2-AR, or their combined activity. Notably, ICI treatment in the absence of Iso produced inverse agonism in TTS, shown by significantly enhanced T50 and decreasing beating rate below baseline (Online Figures 7A to 7C).

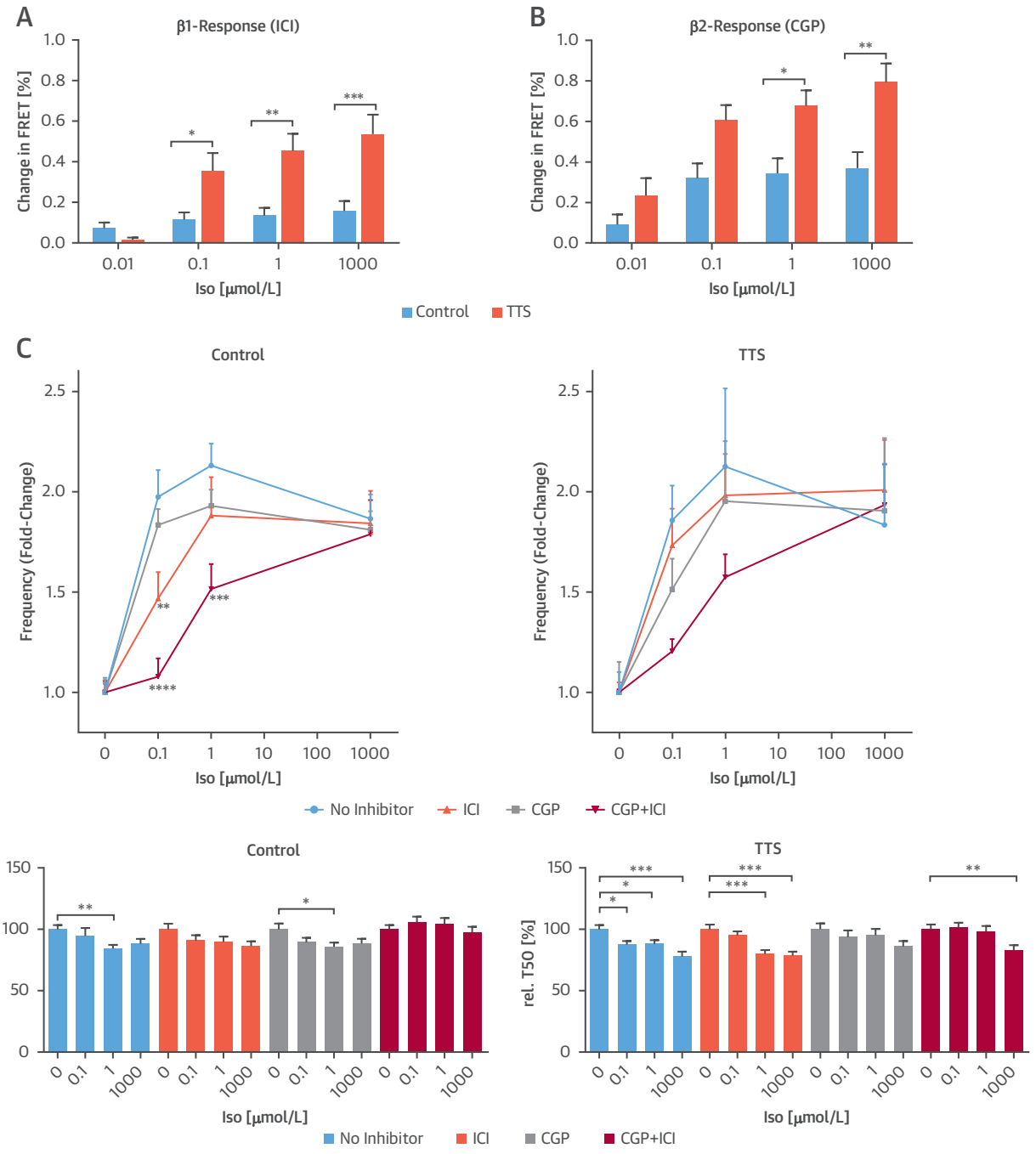
INCREASED LIPID ACCUMULATION AND DECREASED MITOCHONDRIAL AREA IN PATIENTS WITH TTS AFTER CATECHOLAMINE TREATMENT. Adrenergic stimulation results in positive inotropic and chronotropic effects of the heart and regulates lipid metabolism through the PKA pathway and NR4A1 (6,17). Therefore, we analyzed cardiac lipids by Oil Red O staining in catecholamine-treated iPSC-CMs. We observed a dose-dependent increase in lipid droplets in TTS-iPSC-CMs, but not in control cells (**Figures 5A and 5B**). The overall lipid droplet accumulation was highest in Epi-treated iPSC-CMs from patients with TTS (5.0- to 5.7-fold) compared with untreated iPSC-CMs. Based on these data, we analyzed the different lipid transporters and found significantly increased expression of the lipid importer *CD36* in TTS-iPSC-CMs under basal conditions (6.1-fold) and upon treatment with 500 μmol/l Epi (7.1-fold) compared with control iPSC-CMs (**Figure 5C**). Furthermore, *CD36* showed a slight increase (1.7- and 1.6-fold) in expression in control iPSC-CMs after Epi stimulation (100 nmol/l and 500 μmol/l) compared with unstimulated conditions (**Figure 5C**). In addition, the lipid translocator *CPT1C* was expressed at significantly lower levels in TTS-iPSC-CMs compared with control iPSC-CMs after catecholamine treatment (**Figure 5D**). Because mitochondrial disorders have been described as potential causes for lipid accumulation and cardiomyopathies, we characterized changes in the mitochondrial network during catecholamine-induced stress in iPSC-CMs. Cells were treated with Iso (100 nmol/l and 5 mmol/l) and costained with the mitochondrial-specific probe MitoSpy Orange (BioLegend, San Diego, California) and the sarcomeric protein α-actinin (**Figure 5F**). Confocal microscopy showed that control cells treated with high Iso dosages showed a significant decrease in mitochondrial area, but a regular α-actinin-positive sarcomeric structure (**Figures 5E and 5F**). In contrast, iPSC-CMs of patients with TTS already revealed a significantly decreased mitochondrial area at basal conditions without Iso treatment (**Figures 5E and 5F**).

GENETIC-BASED CARDIAC DYSFUNCTION IN PATIENTS WITH TTS. We performed whole-exome sequencing (WES) on deoxyribonucleic acid extracted from primary skin fibroblasts of 4 patients with TTS. Because

FIGURE 3 Continued

(A) T50 times (half-maximal decay of calcium signal) of TTS-CMs (n = 148 cells: 2-TTS [n = 26], 5-TTS [n = 60], and 8-TTS [n = 62]) or control CMs (n = 153 cells (2-control [n = 47], 3-control [n = 67], and iFB2 [n = 39]) treated with increasing Iso concentrations. Data are presented as mean ± SEM. **p < 0.01; ***p < 0.001 significant differences by 2-way ANOVA. **(B)** Force of contraction (FOC) of EHM from control (n = 15 EHMs [2-control (4 EHMs) and iFB2 (11 EHMs)]) and TTS patients (n = 14 EHMs [1-TTS (3 EHM), 8-TTS (11 EHMs)]). Data are presented as mean ± SEM. *p < 0.05 significant differences by 2-way ANOVA. **(C)** Normalized inotropic response to acute Iso stimulation of EHM from control (**blue line**, n = 11 EHM [2-control (4 EHM), iFB2 (7 EHM)]) and TTS patient (**orange line**, n = 10 EHM [1-TTS (3 EHM), 8-TTS (7 EHM)]) in the absence or presence (**dashed lines**, n = 4 EHM each of iFB2 or 8-TTS) of beta-adrenergic blockade with CGP (300 nmol/l) and ICI (50 nmol/l) or after 24-h pre-incubation Iso treatment (control: **gray line**, n = 5 EHM [2-control (2 EHM) and iFB2 (3 EHM)], TTS: **purple line**, n = 7 EHM [1-TTS (3 EHM) and 8-TTS (4 EHM)]). Half-maximal Iso concentrations (Iso EC_{50}) are indicated by **lines**. Data are presented as mean ± SEM. *p < 0.05 significant differences by extra sum-of-squares *F* test. **(D)** Change in spontaneous beating frequency of control and TTS-EHMs after 24-h pre-incubation of Iso (1 μmol/l). The same EHMs were used as in **(C)**; n = 5 for control and 7 for TTS. Data are presented as mean ± SEM. **p < 0.01 significant differences by Student *t* test. **(E)** Iso-dependent EC_{50} change after 24-h pre-incubation with Iso (1 μmol/l) of EHM from control and TTS patients (fold change to respective untreated group). The same EHMs were used as in **(C)**; n = 11 for control and 10 for TTS, or n = 5 for control and 7 for TTS (24-h Iso pre-incubation). **(B to E,** Online Video 2). Data are presented as mean ± SEM. *p < 0.05; ***p < 0.001 significant differences by 1-way ANOVA. For EHM generation, iPSC-CMs of 1-TTS and 8-TTS, 2-C, and iFB2 were used. CGP = β_1-adrenergic receptor blocker CGP 20712A; EHM = engineered heart muscle; ICI = β_2-adrenergic receptor blocker ICI 118551; T50 = time to transient 50% decay (from maximum); other abbreviations as in **Figures 1 and 2**.

FIGURE 4 Subtype-Specific β-AR Signaling in iPSC-CMs



(A and B) Quantification of FRET experiments after selective treatment with the β-AR-inhibitors ICI (β_2-selective antagonist), CGP (β_1-selective antagonist), or both. β_1-AR activity (ICI) and β_2-AR activity (CGP) were significantly increased in TTS compared with control. A total of 23 to 51 cells (ICI) or 11 to 29 cells (CGP) were used (details are shown in Online Table 5). Data are presented as mean ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ significant differences by 2-way ANOVA per group. **(C)** Spontaneous beating frequency on MEA: TTS and control CMs exhibit increased frequencies when stimulated with increasing Iso doses with a maximum at 1 µmol/l. Control: n = 3 MEA experiments (2-control [n = 2], iFB2 [n = 1]); TTS: n = 3 MEA experiments (2-TTS [n = 1], 5-TTS [n = 1], and 8-TTS [n = 1]). Data are presented as mean ± SEM. **$p < 0.01$; ***$p < 0.001$, significant differences by 2-way ANOVA. **(D)** Relative T50 calcium transient times of control and TTS iPSC-CMs treated with selective β-blockers and increased dosages of Iso. Control: 69 cells (2-control [n = 25], 3-control [n = 22], and iFB2 [n = 22]), TTS: 73 cells (2-TTS [n = 26], 5-TTS [n = 24], and 8-TTS [n = 23]). T50 and beating frequency are more strongly influenced by β_1AR in TTS, whereas β_2ARs are the main receptors for Iso-dependent changes in control. Data are presented as mean ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ significant differences by 1-way ANOVA per group. Simultaneous inhibition of both β-AR subtypes (ICI + CGP) suppressed the beating rate (up to 1 µmol/l Iso in TTS and control) and T50 changes (up to 1 µmol/l Iso in TTS). Abbreviations as in Figures 1 to 3.

the limited patient number prevents a significant association of novel gene(s) with a genetically complex disorder, we focused our WES data analysis on 81 genes previously associated with genetic forms of cardiomyopathies and cardiac arrhythmias. Using specific filter criteria, we identified the following candidate variants. Patient 2-TTS carried the heterozygous c.1040A>G variant in RNA binding motif protein 20 (*RBM20*) predicted to substitute the highly conserved tyrosine at position 347 by cytosine (p.Y347C). This variant is not found in over 121,000 alleles in the ExAC database, excluding that this variant represents a single-nucleotide polymorphism common in the general population. Another heterozygous variant, c.1139G>A (p.R380H), was found in dolichol kinase (*DOLK*; present in 5 of >121,000 in the ExAC database), but was predicted as benign. Patient 5-TTS was found to carry the heterozygous rare variant c.567C>G (p.F189L) in the calsequestrin 2 (*CASQ2*) gene (present in 84 of >121,000 in the ExAC database) that was predicted to be deleterious or damaging. No likely candidate variant was found in the other 2 patients. All candidate variants were confirmed by Sanger sequencing (Online Figure 9). This finding of variants in genes encoding important cardiac proteins associated with cardiac pathologies may underlie the hypothesis of a predisposition to TTS, but also suggests a number of unrelated mechanisms underlying the observed phenotypes.

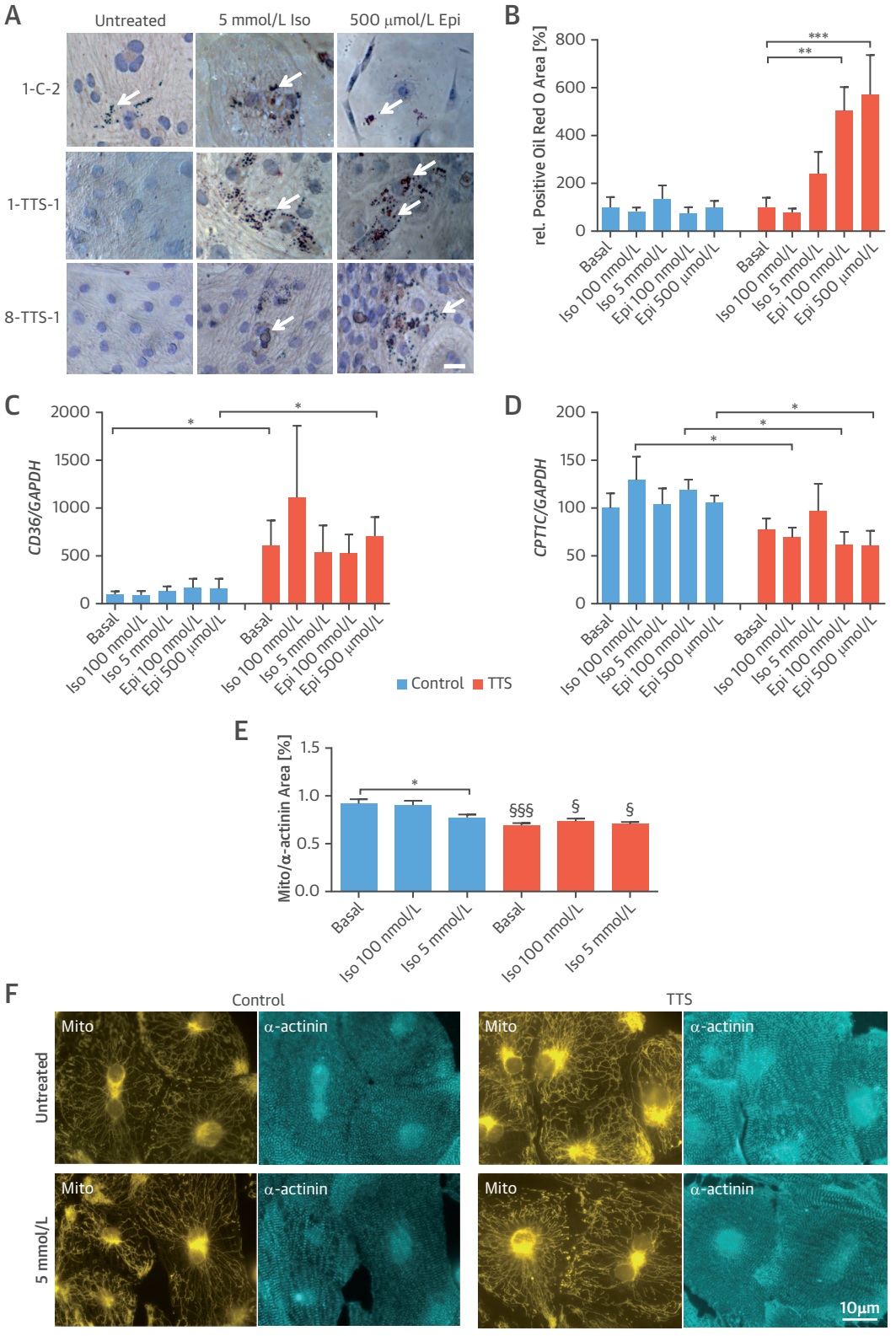
DISCUSSION

Here we demonstrate, for the first time, the feasibility of a human in vitro TTS model by using iPSC-CMs, and we provide evidence that catecholamine-treated TTS-specific iPSC-CMs mimic features consistent with those found in individuals with TTS. Notably, we found that: 1) sudden stress can be induced in iPSC-CMs by short-term catecholamine exposure; 2) β-adrenergic signaling, including cAMP response and cAMP-dependent PKA activity, is increased in TTS-iPSC-CMs after treatment with high levels of catecholamines; 3) β-AR activity is mediated by both β_1- and β_2-ARs in TTS-iPSC-CMs; 4) the active force of contraction is reduced and sensitivity to isoprenaline-stimulated inotropy is enhanced in TTS-EHMs; and 5) electrical activity and lipid accumulation is altered in TTS-iPSC-CMs. In conjunction with the identified genetic variants of several key regulators of cardiac function, our findings suggest a genetic predisposition in TTS. Therefore, iPSC-CMs can overcome the limitations of artificial animal or in vitro TTS models.

The hormone receptor NR4A1 plays a crucial role in cardiovascular diseases and, as such, is markedly expressed in the human heart and in neonatal rat cardiomyocytes after β-adrenergic stimulation, with a proposed protective role in β-AR–induced cardiac remodeling and hypertrophy (18,19). Thus, in our study, we found mRNA expression to be highly up-regulated after exposure to catecholamines in all iPSC-CMs, with a significant increase in patients with TTS under Iso compared with control subjects (**Figure 1E**). β-AR inhibition resulted in clear reduction of NR4A1 protein (Online Figure 8), confirming the dependence of NR4A1 expression on β-AR activity. In addition, it is known that NR4A1 regulates lipid metabolism in muscle cells via modulation of the expression of lipid transporter proteins, such as CD36 (17), which we found to be expressed at higher abundance in TTS (**Figure 5C**). These data argue for NR4A1-dependent crosstalk between the β-AR signaling and lipid metabolism, and suggest NR4A1 as a potential therapeutic target in TTS. However, the exact functional role of NR4A1 in β-AR signaling and lipid homeostasis in the TTS heart remains to be elucidated.

The FRET measurements showed a higher cAMP response to increasing catecholamine levels in TTS-iPSC-CMs compared with control cells (**Figure 2C**). This may result in a cAMP-dependent increased activation of PKA and subsequent specific phosphorylation of its cellular targets, including RYR2-2808, PLN-S16, TNI-S23/24, and Cav1.2-S1928 (**Figures 2D to 2H**). Besides cAMP levels, Iso-dependent beating frequency also shows a larger dynamic range in TTS compared with control subjects (**Figure 2J**). The combined monolayer data suggests increased susceptibility to catecholamines under intensive stress conditions in patients with TTS. This conclusion is strengthened by our EHM data, which demonstrated enhanced sensitivity to Iso in TTS (**Figure 3D**). A possible explanation could be reduced receptor desensitization in TTS after excessive stress. This hypothesis is supported by a less-pronounced right shift of the acute Iso concentration curve after 24-h chronic stimulation in TTS-EHMs in comparison with control EHMs, indicating a lower degree of catecholamine desensitization in TTS-EHMs (**Figures 3C and 3E**). Preliminary data of increased β_1-AR gene expression after catecholamine treatment in TTS compared with control subjects underline the reduced receptor desensitization in TTS (data not shown). As such, a role for β-AR desensitization in cardiac diseases and maladaptive responses has been discussed (20). Furthermore, and consistent with the often-observed neuropsychiatric

FIGURE 5 **Intramyocardial Lipid Accumulation in TTS-iPSC-CMs After Catecholamine Treatment**



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disorders in patients with TTS, β-AR desensitization can be influenced by neurohormonal stimuli (21). However, whether the ligand affinity of β-ARs is different in TTS compared with control subjects is still unclear and needs to be elucidated in the future.

Moreover, in this study, we describe for the first time that β-AR activity in TTS seems to be mainly mediated by β_1-ARs in combination with β_2-ARs. This is shown by a significantly increased Iso-dependent FRET response, PKA-specific phosphorylation at target sites, and electrical beating activity under ICI or CGP treatment compared with control subjects (**Figure 4,** Online Figure 8). This is an interesting finding, because although patients treated with β-blockers developed TTS (2), β_2-ARs can mitigate the response to β_1-ARs by activation of inhibitory G proteins (7). Thus, an increase in β_2-AR activity would not be expected to lead to a TTS phenotype. However, the exact roles of the different β-ARs in TTS are still controversial, as previous studies showed that β_2-ARs play a significant role in TTS development (7).

Interestingly, despite similar basal cAMP levels, we identified a hyperphosphorylation of the PKA target PLN-S16 in TTS under basal conditions (Online Figure 2, **Figure 2**). This is consistent with a lower absolute T50 time in monolayer TTS iPSC-CMs and TTS-EHMs, and an increase in the amplitude of calcium transients in TTS iPSC-CMs under the same conditions (**Figure 3A,** Online Figures 5D and 6A). These data indicate faster reuptake of calcium in the sarcoplasmic reticulum by the SERCA-PLN complex and accelerated relaxation. A possible PKA-independent mechanism for PLN-S16 phosphorylation is mediated by the cyclic guanosine monophosphate (cGMP)-dependent protein kinase I (22). Crosstalk between cGMP and cAMP is mainly mediated by β_3-ARs (23). The contribution of β_3-ARs to the development of TTS and the level of cGMP as a regulator of cAMP in TTS need to be analyzed in the future.

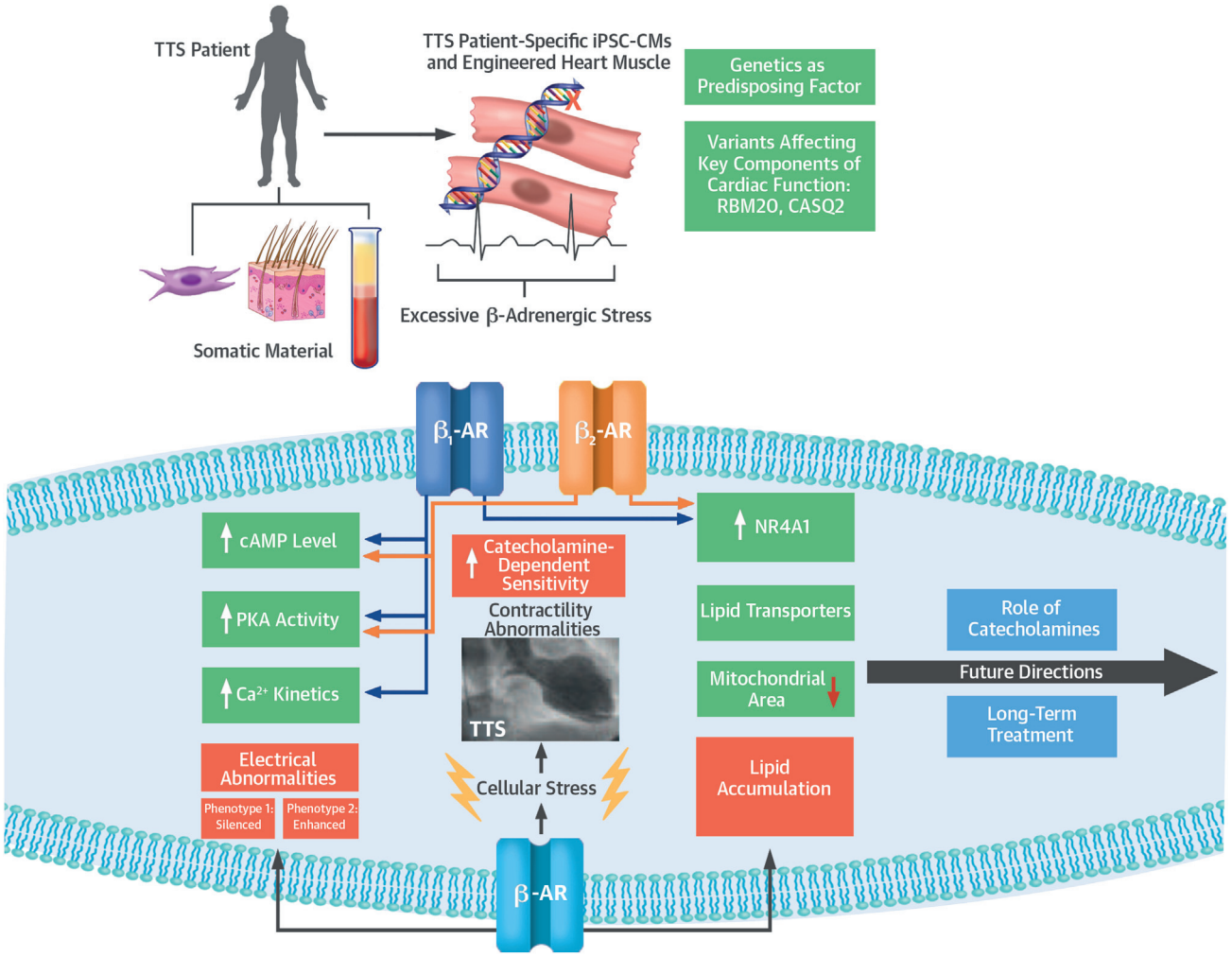
We also identified increased lipid accumulation in catecholamine-treated TTS-iPSC-CMs compared with control iPSC-CMs, which was confirmed by higher expression of the lipid importer *CD36* in TTS-iPSC-CMs and by decreased expression of the *CPT1C* lipid translocase (**Figures 5A to 5D**). This, in fact, suggests that more lipids are imported into the cell, with lower levels of lipid translocation from the cytoplasm into the mitochondria in TTS-CMs. These findings are consistent with previous studies showing Iso-induced lipid accumulation in human biopsies from patients with TTS and in ISO/patient serum-stressed HL-1 cardiomyocytes (6,24). However, the expression of lipid transporters, such as CD36, under acute stress is still controversial (6). As alterations in the activity of proteins, such as CD36 or CPT1C, that are involved in the transport of mitochondrial substrates were described as mitochondrial disorders causing lipid accumulation (25), we analyzed mitochondrial area in iPSC-CMs and found a reduction in TTS compared with control subjects (**Figures 5E and 5F**). The decreased mitochondrial content might influence lipid droplet concentration in TTS. Excessive lipid accumulation may result in lipid peroxidation and disruption of biomembranes, resulting in disturbance of ion transport processes and therefore lipotoxicity (26). Consequently, our observation of increased lipid accumulation after stress in TTS-iPSC-CMs suggests that catecholamine-induced cardiac lipotoxicity contributes to the pathogenesis of TTS.

Another important finding includes the altered electrical activity observed in iPSC-CMs of patients with TTS after Iso stimulation. Under increasing Iso concentrations, TTS-iPSC-CMs showed a larger dynamic range than control cells (**Figure 2J**). In line with these acute monolayer CM data, we showed a greater change in beating frequency after chronic Iso pre-incubation in TTS-EHMs compared with control

FIGURE 5 Continued

(A) Representative images of Oil Red O staining in iPSC-CMs from control (1-C-2) and TTS patients (1-TTS-1, 8-TTS-1) 2 h post-Iso or Epi are shown. Arrows indicate lipid accumulations. Scale bar: 25 μm. **(B)** Quantification of areas positive for Oil Red O in control- and TTS-iPSC-CMs. Control: n = 3 to 9 images (1-control [n = 2 to 8], iFB2 [n = 3]); TTS: n = 4 to 14 images (1-TTS [n = 3 to 9]; 8-TTS [n = 1 to 7]). **p < 0.01; ***p < 0.001, significant differences by 1-way ANOVA. **(C–D)** Expression of *CD36* or *CPT1C* mRNA in iPSC-CMs from control subjects or patients with TTS. Control: n = 7 differentiation experiments (1-control [n = 3], 2-control [n = 2], and iFB2 [n = 2]); TTS: n = 7 differentiation experiments (1-TTS [n = 3] and 8-TTS [n = 4]). *p < 0.05 significant differences by Student *t* test. Data are presented as mean ± SEM of the ratio of normalized mRNA to *GAPDH* as housekeeping gene. **(E and F)** Normalized mitochondrial area (stained with MitoSpy Orange [Mito]) to α-actinin in control (n = 46 to 54 images [1-control (n = 25 to 29), iFB2 (n = 21 to 25)]) and TTS iPSC-CMs (n = 70 to 80 images [1-TTS (n = 23 to 26), 5-TTS (n = 23 to 28), and 8-TTS (n = 24 to 28)]). *p < 0.05; §§§p < 0.001 significant differences by 1-way ANOVA combined with the Student *t* test (§) between groups. **(F)** iPSC-CMs were stained with MitoSpy combined with antibodies against the sarcomeric protein α-actinin with or without catecholamine treatment. Scale bar: 10 μm. Representative images are shown from control and TTS cell lines. *CPT1C* = carnitine palmitoyltransferase 1C; other abbreviations as in **Figure 1**.

CENTRAL ILLUSTRATION **Overactive β-Adrenergic Signaling in TTS-Specific iPSC-CMs**



Borchert, T. et al. J Am Coll Cardiol. 2017;70(8):975-91.

Generated TTS iPSC-CMs show a larger dynamic range toward excessive β-AR stress compared with control cells, as shown by FRET-measured cAMP, PKA activity, and altered Ca^{2+} kinetics resulting in increased electrical abnormalities. The β-AR activity is mainly mediated by β_1-AR in combination with β_2-AR. The induced high β-activation–dependent cellular stress can cause lipid-accumulation–dependent electrical stunning in a rising number of cells. Engineered human myocardium from TTS-iPSC-CMs showed contractility abnormalities such as an impaired force of contraction and a greater sensitivity to catecholamine-induced toxicity as potential mechanisms associated with the TTS phenotype. Taken together with the identified genetic variants in cardiac key regulator genes (*RBM20, CASQ2*), our findings imply a genetic predisposition for TTS with substantial risk under excessive adrenergic stress. Furthermore, our findings shed new light on the possible underlying mechanism of the contractile phenotype. **Red** indicates the main outcomes of this study, such as increased catecholamine-dependent sensitivity, electrical abnormalities, and lipid accumulation. **Green** indicates new players or hints with evidence from this study, and **blue** indicates central issues in TTS, but without reasonable evidence from studies until now. **White and red arrows** indicate differences with control cells. AR = adrenergic receptor; Ca^{2+} = calcium ions; cAMP = cyclic adenosine monophosphate; *CASQ2* = calsequestrin 2; CM = cardiomyocyte; iPSC = induced pluripotent stem cell; PKA = protein kinase A; *RBM20* = RNA Binding Motif Protein 20; TTS = Takotsubo syndrome.

subjects (**Figure 3D,** Online Figure 6B). This higher responsiveness to Iso could be interpreted as a surrogate for the hypercontractile phenotype of basal CMs in the acute phase of TTS. The beating frequency of iPSC-CMs from TTS recorded by MEA was gradually lowered at Iso dosages of 1 μmol/l to 5 mmol/l, which is similar to the range of Iso dosages that resulted in intracellular lipid accumulation as a potential cause of lipotoxicity. This gradual inhibition of electrical activity is in line with earlier findings in HL1-CMs (6).

Another type of TTS-iPSC-CMs in our experiments showed reversible silenced electrical activity, directly after exposure to Iso (**Figure 2I,** Online Figure 4). These findings of a gradual decrease of electrical activity upon high Iso doses and reduced calcium-dependent force generation in TTS-EHMs compared with control subjects (**Figure 2B,** Online Figure 6A) suggest that TTS-iPSC-CMs could recapitulate the acute apical cardiodepression frequently observed in patients with TTS.

We also identified highly relevant variants in cardiac genes, such as *RBM20,* encoding RNA-binding motif protein 20, or *CASQ2*, encoding calsequestrin 2, in patients TTS 2 and 5. Interestingly, autosomal dominant mutations in *RBM20* have been associated with dilated cardiomyopathy (CMD1DD, OMIM 613172). Sequence analyses revealed that the identified *RBM20* variant substitutes the highly conserved tyrosine at position 347 with cytosine (p.Y347C) (Online Figure 9B). It was previously shown by us and others that single variants in conserved regions of RBM20 can lead to mis-splicing of targets, such as titin or CAMKII, and to decreased force of contraction as a cause of dilated cardiomyopathy in 2- and 3-dimensional iPSC-CM heart models (27) (our unpublished data, Streckfuss-Bömeke et al., May 2017). These findings further support the suggested functional evidence of variants in RBM20 in TTS. Autosomal recessive mutations in *CASQ2* have been associated with catecholaminergic polymorphic ventricular tachycardia, with or without structural heart disease (OMIM 611938 and 615441). The *CASQ2* variant F189L is located in a very conserved region (Online Figure 9B), and was shown to have functional consequences for cytosolic Ca^{2+} and human ether-a-go-go-related channels (hERG), supporting the suggested functional evidence on this variant in TTS (28). Variants in hERG itself are also associated with TTS (29). Follow-up studies are required to investigate in detail the effect of the identified novel *RBM20* and *CASQ2* variants on the TTS phenotype. Still, further WES experiments and analysis, especially in larger patient cohorts, will be necessary to prove the hypothesis of a genetic predisposition to TTS. Nevertheless, identifying variants in genes encoding important cardiac proteins associated with cardiac pathologies in 2 of 4 patients suggests that genetic variants affecting different key components of cardiac function may underlie or predispose to TTS. Of note, besides the hypothesized genetic predisposition to TTS, epigenetic mechanisms were shown to be important for the development of stress-induced heart failure (30).

Taken together, based on our results, TTS-CMs show a greater sensitivity and response toward catecholamine stimulation compared with control cells, as shown by FRET-measured cAMP and PKA activity, resulting in enhanced beating frequency and accelerated Ca^{2+} kinetics. This high β-activation-dependent cell stress is mainly mediated by β_1-ARs, and can cause lipid-accumulation-dependent electrical stunning in a rising number of cells, shown by decreasing frequency at excessive Iso dosages simulating an overload in TTS. Furthermore, our data demonstrate that lowering of frequencies at high Iso dosages is also present in control iPSC-CMs, suggesting that TTS might, in principle, be inducible in all humans, whereas patients with TTS are genetically predisposed and at high risk under excessive adrenergic stress (**Central Illustration**).

STUDY LIMITATIONS. It is hypothesized that TTS is based on multiple factors in a complex whole organ setting, rather than on cell-autonomous defects. Therefore, in the future, protocols to differentiate iPSCs into organoid cultures composed of several distinct cell types should be developed to recapitulate features of tissues, rather than that of individual cell types in vitro (31). For this study, we used 2 different selected clones from each TTS patient based on cardiac differentiation capacity and cardiac phenotype. As the genetic background, sex, and age of the patients with TTS and control subjects, as well as the maturity of iPSC-CMs can influence the results of this work, age-matched control iPSCs or healthy family members would be ideal controls and should be considered in future studies. However, donor-specific analysis of various readouts (e.g., calcium T50) showed minor differences between single patients or control subjects (Online Figures 5C and 10). As the contribution of the identified novel *RBM20* and *CASQ2* variants to the observed TTS phenotype is still unclear, detailed functional analyses, including comprehensive genome editing work on these variants, are required in the future. Apart from the studied catecholamines, further investigation of potential protective mechanisms, including estrogen and other signaling, and therapeutic substances besides beta-blockers need to be investigated.

CONCLUSIONS

Our data show that iPSC-CMs from patients with TTS are substantially different from control iPSC-CMs regarding catecholamine susceptibility, β-adrenergic signaling, lipid accumulation, electrophysiology, and active force generation. In conjunction with our

observations regarding the genetic variants of several key regulators of cardiac function, our findings suggest a genetic predisposition in TTS. Furthermore, our findings shed new light on the possible underlying mechanism of the contractile phenotype.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: IPSC-CMs from patients with a severe TTS phenotype show strong catecholamine sensitivity and altered β-adrenergic signaling as a potential disease cause for TTS. Taken together with the identified genetic variants in key cardiac regulator genes, our findings imply a genetic predisposition in TTS through increased sensitivity to catecholamine toxicity.

TRANSLATIONAL OUTLOOK: Further studies, including clinical studies, are needed to establish the detailed mechanism by which higher sensitivity of TTS to Iso, and therefore altered β-adrenergic signaling, results in cardiac dysfunction in patients with TTS. This should help to develop a therapeutic long-term treatment option and pave the way to studying predisposing genetic factors in a large patient cohort.

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KEY WORDS broken heart syndrome, catecholamine, electrical activity, iPSC cardiomyocytes, lipotoxicity, TTS pathogenesis

APPENDIX For an expanded Methods section, supplemental tables and figures, and supplemental videos and their legends, please see the online version of this article.